Hydrogen-Bond Detection in Peptides by ^{1}H-Nuclear Magnetic Resonance through a Hydrogen–Chlorine Exchange Reaction

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NMR spectroscopy is a versatile method for the conformational analysis of peptides and proteins. The hydrogen–chlorine exchange of amide N*H* protons is detected by ^{1}H NMR and used as a method to distinguish between intramolecularly hydrogen-bonded and solvent-exposed N*H* moieties. The method has been applied to hydrogen bond detection in naturally occurring antibiotic peptides, such as gramicidin S, and CH_3CONH–X (X=alkyl- or aryl-) derivatives. The deuterium exchange method was compared with this method in parallel experiments. In the case of chlorine exchange, in contrast to deuterium exchange, the hydrogen-bonded amide protons are replaced much faster than their solvent-exposed counterparts and the duration of the experiments is considerably less. It is highly possible that the hydrogen–chlorine exchange reaction under the present experimental conditions, in the dark and at room temperature, proceeds through an electrophilic polar mechanism.

Several methods have been developed to study the conformation of peptides and proteins in solution. Of the most versatile techniques used for this purpose, NMR-based conformational analysis is distinguished for several reasons, among which are ease of manipulation and rapidity.

In this respect, one of the applications of NMR spectroscopy in the conformational analysis of peptides and proteins is related to amide N*H* protons in peptide molecules. Using ^{1}H NMR methods, N*H* protons can be identified in two categories: those shielded from and those exposed to the solvent. Based on this apparently simple but inherently complex concept, several techniques were designed to differentiate between these amide protons. Among the most important of these techniques are (i) tritium- and deuterium-exchange methods,[1—4] (ii) methods based on the temperature and solvent dependence of N*H* proton chemical shifts,[5,6] and (iii) methods based on the broadening of peptide N*H* resonances on addition of certain radicals.[7]

Many of the N*H* protons in peptides and proteins are hydrogen-bonded intramolecularly. These intramolecular bonds cause complete or partial shielding of the amide protons from the solvent and other environmental forces. Such an observation is utilized effectively in the above-mentioned analytical methods. In this article, we propose a new method capable of detecting intramolecular hydrogen bonds in peptides using ^{1}H NMR spectroscopy.

We have previously reported on this method known as the "hydrogen–chlorine (H–Cl) replacement (exchange) method".[8—10] The replacement reaction of amide protons is initiated by adding peptides to solutions containing Cl_2 or by adding *t*-butyl hypochlorite (*t*-BuOCl) to peptide solutions (Chart 1). The experiments are performed in a dark environment and at room temperature to prevent radical formation as much as possible.

Similar to the tritium and deuterium exchange methods, the rates of chlorination are followed by ^{1}H NMR spectra and the difference between intramolecularly hydrogen-bonded amide protons and those more exposed to solvent and environmental forces can be distinguished clearly and rapidly. The H–Cl exchange method has been successfully applied to several conformational analyses of peptides. These applications and part of the organic chemistry involved are discussed here.

Results and Discussion

Applications of the Hydrogen–Chlorine Exchange Method. The H–Cl exchange method, using ^{1}H NMR, has been applied to detect hydrogen-bonding patterns of certain peptides in solution. The method was compared to the deuterium-exchange method, and in all of the performed experiments, compatible results were observed. The main difference between these methods is that solvent-exposed or weakly hydrogen-bonded amide protons are more labile in deuterium exchange. Interestingly, the H–Cl exchange is more facile and rapid with solvent-shielded or strongly hydrogen-bonded amide protons.

In a previous NMR-aided conformational analysis of gramicidin S (GS) (Fig. 1), a cyclic decapeptide antibiotic isolated from *Bacillus brevis*, the four intramolecular hydrogen bonds between the two pairs of

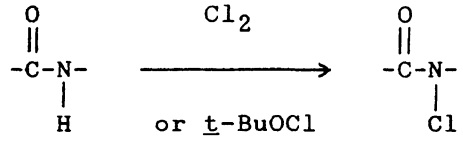

Chart 1.

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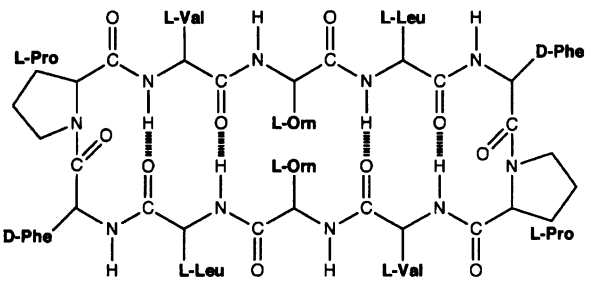

Fig. 1. The Structure of gramicidin S.

Val and Leu residues were detected by the H–Cl exchange method,[8,9,11] and further reconfirmed by temperature-dependent NMR studies.[12] In Fig. 2, the exchange accessibility of the amide protons of GS·2HCl in deuterium or chlorine-containing medium is plotted against time. Figure 2A shows the results for the H–D exchange method. Val N*H* and Leu N*H* protons, which are intramolecularly hydrogen-bonded to further stabilize the GS β-sheet structure, show slower deuterium exchange than the less shielded Orn N*H* and Phe N*H* protons. Figure 2B shows the results for the H–Cl replacement method. The results in Fig. 2A are reversed here. Compared with the amide protons of Orn and Phe, Val and Leu amide protons are substituted much more rapidly. Among the four amino acid residues mentioned above, the Val amide protons show the slowest H–D exchange and the fastest H–Cl exchange rates (Table 1). As a result, it can be concluded that the Val N*H* moieties in GS are more strongly hydrogen-bonded or less solvent exposed than the Leu N*H* moieties. Interestingly, the importance of the hydrogen-bonded Val amide protons, and not those of Leu, for the stability of GS secondary structure and its biological activity has been emphasized.[13,14]

The method was further applied to the detection of hydrogen bonds in the peptide antibiotic tuberactinamine N, and the presence of an intramolecular hydrogen bond in the cyclic part of the molecule[15] was reconfirmed.[9]

The hydrogen bond patterns of the dipeptides Ac–L-Val–Gly–OMe and Boc–L-Val–Gly–OMe, and the tripeptide Boc–Gly–L-Val–Gly–OMe were also rationalized by H–D and H–Cl exchange methods and the results were compared.[8] In the case of Boc–L-Val–Gly–OMe, the results were also supported by temperature-dependent NMR experiments [temperature range: 25—60 °C, 5 °C intervals; solvent: $(CD_3)_2SO$]. The N*H* temperature-dependent shifts of Val and Gly showed a linear behavior, with temperature-dependence coefficients of 8.26×10^{-3} and 3.10×10^{-3} ppm °C^{-1}, respectively.

These studies have so far confirmed the credibility of the mode of behavior of the H–Cl exchange method vs. the more conventional H–D exchange method. In all of these experiments the rate of exchange was faster in the former method.

Organic Chemistry of the Hydrogen–Chlorine Exchange Method. In order to extend the H–Cl replacement reaction to other series of compounds, CH_3CONH–R (or –Ar) as models for the peptide backbone, and to be able to explain the different reactivities of amide protons in the chlorination reaction, further experiments were performed.[10] The amide N*H* proton of *N*-methylacetamide (**1**) was replaced by deuterium or chlorine. The rate of the replacement was estimated by plotting the exchange accessibility of the amide protons against time and the results are shown in Fig. 3 for methanolic solutions. Similar experiments were carried out in $CDCl_3$, CF_3CH_2OH, and $(CD_3)_2SO$.[10]

Table 2 shows the effects of alkyl group (R) substitutions on the rate of the H–Cl replacement reaction in CH_3CONH–R. Electron-withdrawing groups such as cyanomethyl (**2**) and electron-donating groups such as isobutyl (**5**) have opposite effects on the reactivity and chemical shifts. Electron-withdrawing groups increase the positive charge density on amide protons, hence, the H–Cl exchange reaction becomes faster. This electron deshielding effect can serve as a model of hydrogen-bonding for amide proton exchange reactions in peptides.

In other experiments, the behavior of amide protons was investigated in *o*- and *p*-substituted acetanilides. Table 3 summarizes the chemical shift differences ($\Delta\delta$) of these compounds in several solvents. The amide protons of *o*-substituted acetanilides depict small chemical shift differences($\Delta\delta$) for the $CDCl_3$–CH_3OH and $CDCl_3$–$(CD_3)_2SO$ solvent pairs, and this is quite apparent in ethyl *o*-acetamidobenzoate (**12**). The presence of intramolecular hydrogen bonding in these compounds can be supported further by physicochemical data (**11**, mp 110 °C; **12**, mp 61 °C) and more downfield chemical shifts of the amide N*H* protons.

Slow H–D exchanges for the solvent-shielded amide proton of **12**, shown in Table 4, give additional evidence for the existence of intramolecular hydrogen bonding. Concomitantly, the $T/2$ values for chlorination of *ortho* compounds (**12**, **14**, and **16**) are within two minutes and this observation further supports the ease of chlorination in the hydrogen-bonded amide protons. After all, the rapid exchange of the deuterium in the case of compounds **13** and **14** can not be explained clearly; though in the case of chlorine exchange, the exchange rate of **14** is comparable to those of compounds **12** and **16** (Chart 2).

The mechanism of the ring chlorination of anilines and other related aromatic amines, and the *N*-chlorination of benzamides by *t*-BuOCl was discussed in detail.[16,17] The mentioned reactions involve an electrophilic attack by a positive halogen provided by an appropriate halogen source.

On the other hand, detailed studies on the light- or thermally-induced *t*-butyl hypochlorite chlorination of a range of compounds, with different functional groups,

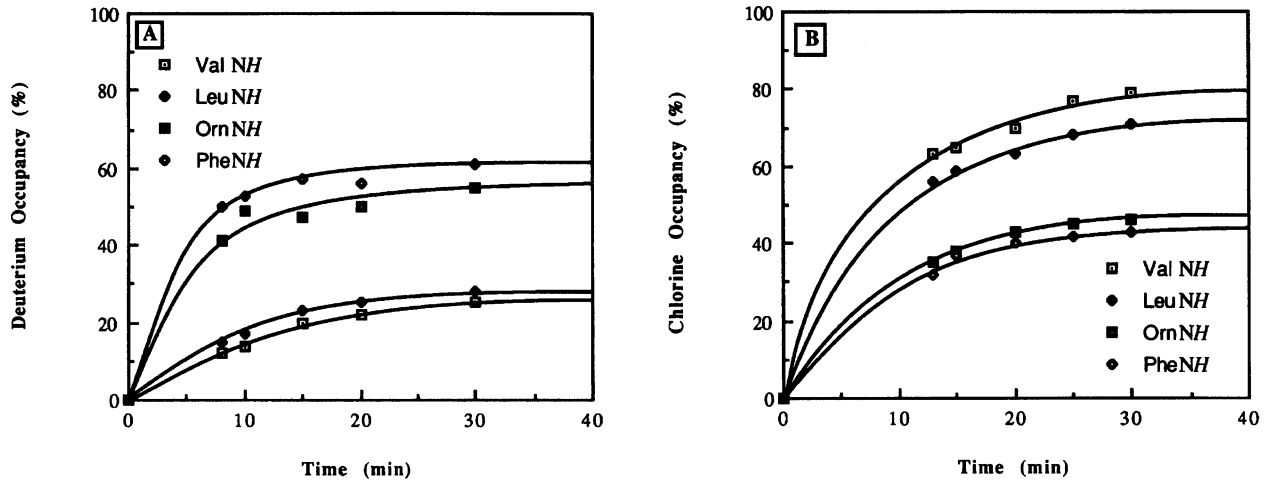

Fig. 2. Comparison of hydrogen–deuterium exchange and hydrogen–chlorine replacement methods for gramicidin S hydrochloride (GS·2HCl) [0.05 M (=0.05 mol dm^{-3} solutions)]: (A) H–D exchange; solvent D_2O (10 equiv of the peptide content)–$(CD_3)_2SO$; $T/2$ values for H–D exchange, L-Val N*H* >24 h, L-Leu N*H*≈21 h, L-Orn N*H* 18 min, D-Phe N*H* 8 min. (B) H–Cl exchange; solvent Cl_2 (19 equiv of the peptide content)–$(CD_3)_2SO$; $T/2$ values for H–Cl replacement, L-Val N*H* 8 min, L-Leu N*H* 11 min, L-Orn N*H*≈100 min, D-Phe N*H*≈115 min.

Table 1. Approximate Rates ($T/2$) of the H–D Exchange and H–Cl Replacement Reactions in Peptide N*H* Protons of Gramicidin S (0.05 M) in Different Solvents
(The calculated equivalents shown in parentheses are based on the content of the peptide.)

Solvent	L-Val	L-Leu	L-Orn	D-Phe
D_2O (10 equiv) in $(CD_3)_2SO$	>24 h	21 h	18 min	8 min
CD_3OD (20 equiv) in $(CD_3)_2SO$	90 min	70 min	13 min	2 min
Cl_2 (20 equiv) in $(CD_3)_2SO$	7 min	10 min	100 min	120 min
t-BuOCl (60 equiv) in CH_3OH–CF_3CH_2OH (1 : 1)	65 min	110 min	>4 h	>4 h

Table 2. Approximate Rates ($T/2$) of the H–D Exchange and H–Cl Replacement Reactions in Amide N*H* Protons of 0.67 M CH_3CONH–R in $CDCl_3$
(The calculated equivalents shown in parentheses are based on the N*H* content of the compounds.)

No.	–R	δ in ppm	CD_3OD (1 equiv)	CD_3OD (5 equiv)	*t*-BuOCl (1.2 equiv)	*t*-BuOCl (2 equiv)
1	$-CH_3$	6.25	18 min	5 min	10 min	5 min
2	$-CH_2CN$	7.25	2 min	1 min	4 min	3 min
3	$-(CH_2)_2CH_3$	6.10	5 min	2 min	8 min	5 min
4	$-(CH_2)_6CH_3$	6.09	—	9 min	—	4 min
5	$-CH_2CH(CH_3)_2$	6.10	20 min	5 min	20 min	12 min

showed the involvement of radical mechanisms.[18,19] The chlorination mechanism via *t*-BuOCl is also solvent dependent.[19,20]

Our experiments were performed in the dark, at room temperature in the presence of oxygen and in polar solvents. Under these conditions the *t*-alkyl hypochlorites are more stable and less prone to decomposition.

From the above experimental results, we conclude that the chlorination mechanism of N*H*, hydrogen-bonded in the peptide backbone of the discussed compounds, appears to involve an electrophilic attack by chlorine. It is highly possible that the partially positively charged chlorine reacts with the partially negatively-charged nitrogen of the peptide backbone via a polar interaction (polar mechanism) (Fig. 4), though other factors such as steric hindrance around the nitrogen, side chain effects, and the solvent polarity can either encourage or discourage this interaction. As a matter of fact, the possibility of an electrophilic chlorine radical attack can not be dismissed completely, but it does not seem likely to be the predominant reaction pathway under the above-mentioned experimental conditions.

So far in comparison with the conventional meth-

Table 3. Solvent Dependent Behavior of Amide N*H* Chemical Shifts (δ) of CH_3CONH–Ar (0.4 M)

No.	Ar–	δ in ppm			Shift difference, $\Delta\delta$		
		$CDCl_3$ (1)	CH_3OH (2)	$(CD_3)_2SO$ (3)	(1-3)	(1-2)	(2-3)
11	*p*-EtOOC–C_6H_4–	8.25	10.10	10.26	2.01	1.85	0.16
12	*o*-EtOOC–C_6H_4–	11.12	11.12	10.62	0.50	0	0.50
13	*p*-NO_2–C_6H_4–	—	—	10.54	—	—	—
14	*o*-NO_2–C_6H_4–	10.35	10.30	10.27	0.08	0.05	0.03
15	*p*-CH_3O–C_6H_4–	7.89	9.80	9.75	1.86	1.91	0.05
16	*o*-CH_3O–C_6H_4–	7.85	9.10	9.06	1.21	1.25	0.04

Table 4. Approximate Rates ($T/2$) of the H–D Exchange and H–Cl Replacement Reactions in Amide N*H* Protons of 0.4 M CH_3CONH–Ar in $(CD_3)_2SO$ (The calculated equivalents shown in parentheses are based on the N*H* content of the compounds.)

No.	Ar–	δ for –N*H* in ppm	D_2O (1.5 equiv)	D_2O (5 equiv)	Cl_2 (1 equiv)
11	*p*-EtOOC–C_6H_4–	10.26	60 min	2 min	60 min
12	*o*-EtOOC–C_6H_4–	10.62	200 min	22 min	2 min
13	*p*-NO_2–C_6H_4–	10.54	1 min	1 min	20 min
14	*o*-NO_2–C_6H_4–	10.27	1 min	1 min	2 min
15	*p*-CH_3O–C_6H_4–	9.75	18 min	—	8 min
16	*o*-CH_3O–C_6H_4–	9.06	21 min	—	2 min

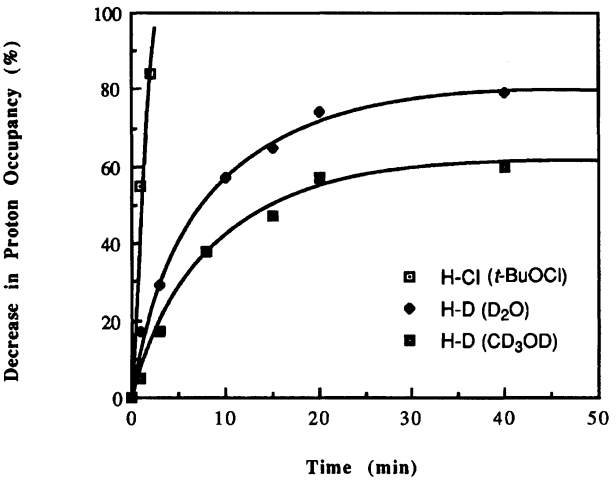

Fig. 3. Comparison of hydrogen–deuterium exchange and hydrogen–chlorine replacement methods for $CH_3CONHCH_3$ amide proton (0.5 M solutions): solvent CD_3OD (5 equiv of the compound's N*H* content)–CH_3OH, $T/2$ 16 min (■); solvent D_2O (5 equiv of the compound's N*H* content)–CH_3OH, $T/2$ 8 min (◆); solvent $(CH_3)_3COCl$ (5 equiv of the compound's N*H* content)–CH_3OH, $T/2<1$ min (⊡).

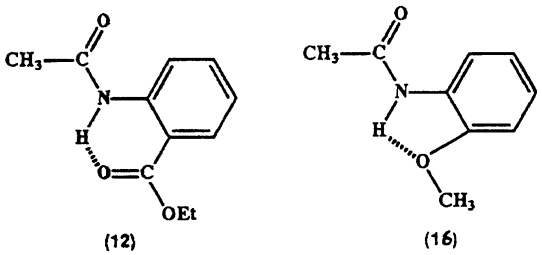

Chart 2.

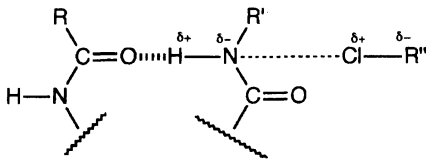

Fig. 4. A possible mechanism for the H–Cl exchange reaction (R, R′=peptide moieties or other parts of the molecules, R″=Cl or *t*-BuO).

ods, the H–Cl exchange method for detecting hydrogen bonds in peptides, has been less time consuming. However, as a distant goal, extension of this method to the conformational analysis of peptides and proteins requires more detailed experiments.

Experimental

General. The reported melting points are not corrected. ^{1}H NMR spectra were recorded by a JEOL-JNM-MH 100 spectrophotometer, with tetramethylsilane as the internal standard. Compounds **1,13,14,15** and **16**, and the starting amino derivatives used for the syntheses of compounds **2,3,4,5,11**, and **12** were commercially available as guaranteed reagents from Wako Chemical Industries Ltd. and Tokyo Kasei Organic Chemicals. *t*-BuOCl was of extra-pure grade from Tokyo Kasei. Dry chlorine gas was produced in a small scale apparatus from the action of concentrated hydrochloric acid on potassium permanganate. All of the solvents utilized in the NMR probes were of NMR spectroscopic grade from Aldrich. Compounds **2,3,4,5,11**, and **12** were synthesized from their corresponding amino derivatives through acylation with acetic anhydride in pyridine. Their purities and structures were further confirmed by TLC

experiments on silica gel GF_{254} (Merck) and ^1H NMR measurements.

***N*-Cyanomethylacetamide (2).** Oil; ^1H NMR ($CDCl_3$) δ=7.25 (bs, 1H, N*H*), 4.21 (bm, 2H, CH_2), and 2.04 (s, 3H, CH_3CO).

***N*-Propylacetamide (3).** Oil; ^1H NMR ($CDCl_3$) δ=6.10 (bs, 1H, N*H*), 3.19 (q, 2H, NHCH_2), 1.97 (s, 3H, CH_3CO), 1.50 (q, 2H, CH_2CH$_3$), and 0.89 (t, 3H, CH$_2$CH_3).

***N*-Heptylacetamide (4).** Oil; ^1H NMR ($CDCl_3$) δ=6.09 (bs, 1H, N*H*), 3.21 (m, 2H, NHCH_2), 1.95 (s, 3H, CH_3CO), 1.27 (bm, 10H, 5×CH_2), and 0.87 (s, 3H, CH$_2$CH_3).

***N*-Isobutylacetamide (5).** Oil; ^1H NMR ($CDCl_3$) δ=6.10 (s, 1H, N*H*), 3.52 (d, 2H, CH_2), 2.39 (s, 3H, CH_3CO), 1.92 (m, 1H, C*H*), and 0.90 (d, 6H, 2×CH_3);

Ethyl *p*-Acetamidobenzoate (11). Mp 110 °C [lit,[21] 103—104 °C]; ^1H NMR ($CDCl_3$) δ=8.25 (bs, 1H, N*H*), 7.92 (d, 2H, *Ar*), 7.55 (d, 2H, *Ar*), 4.51 (q, 2H, CH_2CH$_3$), 2.16 (s, 3H, CH_3CO), and 1.35 (t, 3H, CH$_2$CH_3).

Ethyl *o*-Acetamidobenzoate (12). Mp 61 °C [lit,[21] 66 °C]; ^1H NMR ($CDCl_3$) δ=11.12 (bs, 1H, N*H*), 8.72 (s, 1H, *Ar*), 8.05 (s, 1H, *Ar*), 7.54 (t, 1H, *Ar*), 7.07 (t, 1H, *Ar*), 4.38 (q, 2H, CH_2CH$_3$), 2.22 (s, 3H, CH_3CO), and 1.40 (t, 3H, CH$_2$CH_3).

NMR Measurements. NMR probe samples for the H–Cl exchange experiments were prepared in the dark and at room temperature:

(a) by dissolving the appropriate equivalent of dry chlorine in NMR solvents [0.4 mL (L=dm^3)] at 0 °C (the exact amount of the dissolved gas was determined by weighing the sample tube before and after the addition of chlorine) and adding the related compounds (0.016—0.020 mmol) to this solution or,

(b) by adding the appropriate equivalent of fresh *t*-BuOCl (0.113 mL mmol^{-1}) to previously prepared solutions of the related compounds (0.016—0.020 mmol in 0.4 mL of solvent).

NMR probe samples for the H–D exchange experiments were prepared at room temperature by adding the appropiate equivalent of a deuterated agent (D_2O or CD_3OD) to the peptide solutions.

Four solvents, CH_3OH, CF_3CH_2OH, $CDCl_3$, and $(CD_3)_2SO$, were used in the H–Cl exchange experiments. $CDCl_3$ and $(CD_3)_2SO$ proved to be more appropriate in the replacement experiments performed with Cl_2, whereas CH_3OH, CF_3CH_2OH, and $CDCl_3$ were suitable solvents for *t*-BuOCl. CH_3OH was not appropriate for Cl_2 because O*H* resonances shifted downfield and overlapped with those of peptide N*H* moieties. $(CD_3)_2SO$ did not show good results for *t*-BuOCl as the progress of the displacement reaction in this solvent was undetectable.

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